Food Chemistry 196 (2016) 1–8

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Multiresidue analysis of multiclass pesticides and polyaromatic hydrocarbons in fatty fish by gas chromatography tandem mass spectrometry and evaluation of matrix effect

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ARTICLE INFO

Article history:
Received 9 July 2014
Received in revised form 14 July 2015
Accepted 5 September 2015
Available online 8 September 2015

Keywords:
Fatty fish
Pesticide residues
Polyaromatic hydrocarbons
Sample preparation
Gas chromatography mass spectrometry
Matrix effect

ABSTRACT

This paper reports a selective and sensitive method for multiresidue determination of 119 chemical residues including pesticides and polyaromatic hydrocarbons (PAH) in high fatty fish matrix. The novel sample preparation method involved extraction of the target analytes from homogenized fish meat (5 g) in acetonitrile (15 mL, 1% acetic acid) after three-phase partitioning with hexane (2 mL) and the remaining aqueous layer. An aliquot (1.5 mL) of the acetonitrile layer was aspirated and subjected to two-stage dispersive solid phase extraction (dSPE) cleanup and the residues were finally estimated by gas chromatography mass spectrometry with selected reaction monitoring (GC–MS/MS). The co-eluted matrix components were identified on the basis of their accurate mass by GC with quadrupole time of flight MS. Addition of hexane during extraction and optimized dSPE cleanup significantly minimized the matrix effects. Recoveries at 10, 25 and 50 μg/kg were within 60–120% with associated precision, RSD < 11%.

1. Introduction

Recent studies on marine pollution have reported the presence of multiclass organic contaminants in coastal water as a consequence of the diverse range of anthropogenic activities within their watersheds (Munaron, Tapie, Budzinski, Andral, & Gonzalez, 2012; Sapozhnikova, Wirth, Schiff, Brown, & Fulton, 2007). Chemotherapeutants such as certain organophosphate, carbamate and pyrethroid pesticides are being used in coastal aquaculture to manage pest and disease infestations (Rico et al., 2012). In addition, the marine environment is subject to indirect fluxes of pesticides from widespread agricultural use in nearby crops (García-Rodríguez, Cela-Torrijos, Lorenzo-Ferreira, & Carro-Díaz, 2012). Lipophilic organic contaminants of traditional concern such as organochlorine pesticides (OCs) and polycyclic aromatic hydrocarbons (PAHs) have been monitored widely in fish tissue and the marine environment (Sarkar et al., 2008). In addition to OCs and PAHs, several other classes of pesticides are becoming a point of concern because of their potential bioaccumulation in fish tissue (Chen et al., 2009).

Between 2012 and 2013, India exported 928,215 tons of marine produce with a value of $3.5 billion, which increased by 7.68% this year. The marine products from India are mainly exported to South East Asia (23.12% of the total export), the European Union (22.14%), USA (21.29%), Japan (10.61%), China (7.67%) and the Middle East (5.96%) (http://www.mpeda.com/inner_home.asp?pg=trends). Food safety regulations are becoming increasingly stringent worldwide. Japan has specified Maximum Residue Limits (MRL) for a diverse range of pesticides and contaminants (http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/MRLs-p). The EU legislation prohibits the presence of pesticide residues in fish and fishery products, although currently there is no specific MRL recommended (ec.europa.eu/sanco_pesticides/public/index.cfm). The regulations are similar in most other countries, and MRLs will likely be enforced in these countries in the near future (http://www.cbi.eu/system/files/marketintel/Germany_legisltion_MRLs_in_fishery_products_additional_requirements.pdf).

Marine fishes inherently have high lipid content. During sample preparation, these lipid components often get co-extracted and interfere with the detection and quantification of target analytes by GC–MS. Several approaches have been reported to eliminate these matrix interferences, such as methodologies involving liquid–liquid partitioning, gel permeation chromatography, column

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http://dx.doi.org/10.1016/j.foodchem.2015.09.014
0308-8146/

chromatography, multi-stage cleanup, and low temperature cleanup (LeDoux, 2011). However these methods are time consuming and labor intensive. So far, there is limited literature available on applications of QuEChERS methodology in fish matrices that include analysis of pyrethrins and pyrethroids and a multiresidue method for 13 pesticides in fish muscle (Lazartigues et al., 2011; Rawn, Judge, & Roscoe, 2010). Recently, a QuEChERS based method was reported for the analysis of 13 flame retardants, 18 pesticides, 14 polychlorinated biphenyl (PCB) congeners, 16 polycyclic aromatic hydrocarbons (PAHs), and 7 polybrominated diphenyl ether (PBDE) congeners in catfish muscle, which uses a proprietary zirconium-based sorbent for dispersive solid-phase extraction (dSPE) cleanup and low pressure GC–MS/MS (gas chromatography tandem mass spectrometry) for analysis (Sapozhnikova & Lehotay, 2013). QuEChERS methodology when evaluated for other high fat matrixes such as milk, egg and avocado reported high matrix interference and low recovery particularly for non-polar compounds (~27% for hexachlorobenzene) (Lehotay, Mastovska, & Yun, 2005; Wilkowska & Biziuk, 2011). Hence, at present, very few sample preparation methods deal effectively with the challenges of simultaneous analysis of a varied group of chemical contaminants in fatty fish matrix. So far, even the QuEChERS based multiresidue strategies have targeted only a limited number of compounds in fish matrix, and high matrix effect and low recoveries have been reported for several analytes (Munaretto et al., 2013; Norli, Christiansen, & Deribe, 2011). The increasing international trade of seafood and marine produces makes it necessary to screen for a wide variety of chemical contaminants in these matrices. Considering these deficiencies, we endeavored to develop a sample preparation method for efficient analyses of a diverse range of contaminants in fatty fish matrix. The test analytes were selected covering major OCs and 15 USEPA (United States Environment Protection Agency) priority PAHs. Major GC amenable organophosphates, pyrethroids, carbamate insecticides, herbicides and fungicides were also selected based on their use in aquaculture and agricultural fields. Few more important contaminants in fish like 2,4-D, diuron, dalapon, diquat, paraquat, malachitegreen and methylene blue could not be included in this study since they are not amenable to GC analysis and are mainly estimated by single residue methods. In brief, this work presents a multiresidue method for simultaneous analysis of 22 OCs, 15 PAHs and 82 multiclass pesticides in fatty marine fish meat. The method uses a triple partitioning extraction between water, acetonitrile and hexane followed by a two stage dSPE cleanup to minimize lipid co-extracts prior to GC–MS/MS analysis. Calcium chloride ($CaCl_2$) was used in dSPE cleanup for removal of free fatty acids (Patil et al., 2009). Cobia (*Rachycentron canadum*) meat was used for method standardization. Offshore aquaculture of this marine fatty fish (lipid content ~ 6–11%) is an emerging industry and hence was considered as an ideal or representative matrix for the study (Liu et al., 2009).

2. Experimental

2.1. Chemicals

Certified reference standards of the test analytes (Appendix A) had >98% purity and were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The extraction solvents (*n*-hexane, cyclohexane, ethyl acetate and acetonitrile; all specially dried, residue analysis grade) and other reagents such as sodium acetate (NaAc) and anhydrous calcium chloride ($CaCl_2$) were purchased from Thomas Baker (Mumbai, India). dSPE materials, such as primary secondary amine (PSA, 40 μm, Bondesil), graphitized carbon black (GCB), florisil and C18 were procured from Agilent Technologies (Bangalore, India). Anhydrous sodium sulphate and magnesium sulfate (Analytical Reagent grade) was purchased from Merck (Mumbai, India) and activated by heating at 650 °C for 4 h before use.

2.2. Apparatus

The analyses of samples were performed using a GC equipped with a CTC Combipal auto sampler (CTC Analytics, Switzerland) attached to a triple quadrupole mass spectrometer (GC: 7890A, MS: 7000B, Agilent Technologies, Palo Alto, USA). The system was controlled using Mass Hunter software (ver B.05.00.412). The analytical separation was performed using a VF-5MS (30 m × 0.25 mm, 0.25 μm) capillary column (Agilent Technologies) with mid-point back flush set up for the 15 m column towards the injector port end, for which additional helium flow was supplied through a purged ultimate union. A gooseneck liner (78.5 mm × 6.5 mm, 4 mm) from Restek Corporation (PA, USA) was used. The carrier gas (Helium) flow was set at a constant rate of 1.2 mL/min for the first column, and 1.24 mL/min for the second column. The oven temperature program was set at initial temperature of 70 °C (1 min hold), ramped to 150 °C at 25 °C/min (0 min hold), then at 3 °C/min up to 200 °C (0 min hold) and finally to 285 °C at 8 °C/min (9 min hold) resulting in a total run time of 40.49 min. The transfer line temperature was maintained at 285 °C. During a 3 min post-run period, the oven temperature was maintained at 285 °C with the carrier gas flow rate in column 1 set at −3.4 mL/min.

The multi-mode inlet (MMI) was operated in solvent vent mode and 5 μL of sample was injected. The programmable temperature vaporizer (PTV) program was set at the initial temperature of 70 °C (0.07 min hold), raised to 87 °C at 50 °C/min (0.1 min hold) followed by rapid heating at 700 °C/min up to 280 °C (3 min hold). The purge flow to solvent vent was maintained at 50 mL/min, at a pressure of 11.266 psi until 0.17 min after injection. Next, the split vent was closed for 2.7 min to transfer the analytes to the column. Then, the split vent was opened to remove the high boiling matrix compounds from the inlet. The mass spectrometer was operated in MS/MS mode with acquisition starting at 4.4 min. Electron impact ionization (EI+) was achieved at 70 eV and the ion source temperature was set at 280 °C. The compound specific selective reaction monitoring (SRM) transitions for all the test compounds and other parameters are presented in Appendix A.

2.3. Standard preparation and calibration

Stock standard solutions of each compound were prepared by dissolving 10 ± 0.1 mg of compound in 10 mL acetonitrile and stored in amber colored glass vials at −20 °C. An intermediate mixture at a concentration of 10 mg/L was prepared by diluting an adequate quantity of each stock solution in acetonitrile. A working standard solution (1 mg/L) was prepared by diluting the intermediate standard solution with acetonitrile and storing it at −20 °C. The calibration standards at 5, 10, 25, 50, 100 and 250 μg/L were freshly prepared for daily use from the working standard solution. The calibration curves were prepared by plotting the individual peak areas against the concentration of the corresponding calibration standards in acetonitrile. Matrix matched standards at the same concentrations were simultaneously prepared using a control fish meat extract, previously confirmed for the absence of all the test compounds. For this, the control sample was initially tested by an earlier reported method (Lehotay et al., 2005) and subsequently using the proposed method (described in Section 2.4.3) and the residues were estimated by the instrumental method described in Section 2.2.

2.4. Optimization of sample preparation

2.4.1. Optimization of extraction solvent

Homogenized fish meat (5 g) in a 50 mL centrifuge tube was spiked with the test analyte mix at 50 μg/g and held for 1 h before proceeding with further experiments. Next, 5 mL of distilled water was added, and the tubes were vortexed for 1 min. The residues were extracted in separate batches using three different solvents: (1) 15 mL of ethyl acetate, (2) a combination of cyclohexane + ethyl acetate (9:1, v/v, in presence of 10 g Na_2SO_4) and (3) buffered acetonitrile (+1% acetic acid in presence of 6 g $MgSO_4$ and 1.5 g NaAC) in 6 replicates. dSPE cleanup of the different extracts (1.5 mL) was done with a combination of 50 mg PSA and 50 mg C18 sorbents in addition to 150 mg $MgSO_4$. Recoveries of the test analytes corresponding to different extraction solvents were estimated using solvent-based calibrations and compared.

2.4.2. Optimization of dSPE cleanup

The fish meat used during method optimization contains high amounts of fat (~8%) which partially gets co-extracted in acetonitrile as observed in GC–MS full scan (m/z = 50–500) analysis. For cleanup optimization, the control fish meat (5 g) was extracted with acidified acetonitrile and the extract was spiked at 50 μg/mL level. Cleanup was achieved using different combinations of $CaCl_2$, PSA, florisil and C18 in addition to 150 mg of $MgSO_4$ in Eppendorf tubes, following the optimization of extraction solvent procedure described previously.

Cleaned supernatants from each tube were analyzed by GC–MS/MS. Recoveries of the test analytes from different treatments were compared to finalize the optimum dSPE sorbents. The analyte responses in the case of the cleaned extracts were compared with those obtained in solvent standards. The acetonitrile extracts of the fish matrix before and after the optimized cleanup were also compared in high resolution full scan analysis on a GC-QTOF MS (7200 Q TOF, Agilent Technologies, Bangalore, India) to evaluate the effectiveness of the cleanup procedure.

2.4.3. Final sample preparation method

Approximately 2 kg fish meat was separated from bones and skin and crushed thoroughly in a homogenizer. A subsample of 5 g homogenized meat was weighed into a 50 mL centrifuge tube, mixed with 5 mL of distilled water and vortexed for 1 min. Next, 15 mL of acetonitrile (+1% acetic acid) and 2 mL of hexane were added, and the tube was vortexed again for 1 min. Subsequently, 6 g of $MgSO_4$ and 1.5 g of NaAC were added to each tube, followed by vortexing for 2 min and centrifugation at 5000 rpm for 5 min. A portion of the middle organic layer (1.5 mL acetonitrile) was pipetted out of each tube and kept in a 15 mL centrifuge tube at −20 °C for 20 min. Adsorbents (100 mg $CaCl_2$ + 150 mg $MgSO_4$) were added to the tube for dSPE cleanup. The supernatant (1 mL) was further cleaned with 50 mg PSA + 50 mg florisil + 150 mg C18 + 150 mg $MgSO_4$, vortexed for 1 min and, centrifuged at 10,000 rpm for 5 min. The supernatants from each tube were filtered through a PTFE membrane and analyzed by GC–MS/MS.

2.5. Method validation

The performance of the analytical method was assessed as per the DG-SANCO guidelines for the validation of the analytical methods (Document No. SANCO/10684/2009). The following parameters were considered during the validation process.

2.5.1. Sensitivity

The sensitivity of the method was determined in terms of limit of detection (LOD) and limit of quantification (LOQ) of the test compounds. The LOD was determined by considering a signal to noise ratio (S/N) of 3 with reference to the background noise obtained for an unspiked matrix blank. LOQs were determined by considering a S/N of 10 with the qualifier SRM having S/N ⩾ 3:1.

2.5.2. Matrix effect (ME)

The ME was evaluated by comparing peak areas of the matrix matched standards (peak area of post-extraction spike) with the corresponding peak areas of standards in solvent at 25 μg/kg in ten replicates. The ME was quantified as the average percent suppression or enhancement in the peak area using the following equation:

$$\text{ME}\ (\%) = \frac{\text{Peak area of matrix matched standard} - \text{peak area of solvent standard}) \times 100}{\text{Peak area of matrix matched standard}}$$

A negative value of ME signifies matrix induced signal suppression, whereas a positive value signifies an enhancement in signal intensity.

2.5.3. Accuracy-recovery experiments

The recovery experiments were carried out by spiking the homogenized fish meat (5 g) in six replicates with the test analytes under study at three concentration levels: 10, 25 and 50 μg/kg. These samples were processed following the optimized protocol and analyzed using GC–MS/MS. The quantification was performed using external calibration standards (matrix matched).

2.5.4. Precision

The precision in the conditions of repeatability (three different analysts prepared six samples each on a single day) and the intermediate precision (a single analyst prepared six samples each on three different days) were estimated separately at 25 μg/kg. Precision was expressed as the ratio of the reproducibility standard deviation (RSD_R) to the predicted relative reproducibility standard deviation ($PRSD_R$) and repeatability standard deviation (RSDr) to the predicted repeatability standard deviation (PRSDr) for the assessment of reproducibility and repeatability, respectively. According to Horwitz, the ratio between the calculated and the predicted values should be ⩽2 (known as the HorRat value) (Horwitz & Albert, 2006). This is also applicable for the Thompson equation which suggests that at concentration below 120 μg/kg, $PRSD_R$ = 22.0 and PRSDr = 0.66 $PRSD_R$. The Thompson equation is claimed to be better able to account for the precision at an analyte concentration below120 μg/kg and hence in this study, the Thompson equation was followed (Thompson, 2000).

2.5.5. Assessment of uncertainty

The combined uncertainty was assessed as per the statistical procedure described in EURACHEM/CITAC Guide CG 4 in the same way as reported earlier (http://www.measurementuncertainty.org; Banerjee et al., 2007; Dasgupta et al., 2011). The following variables were evaluated for all the test compounds: uncertainty associated with the calibration graph (u_1), day-wise uncertainty associated with precision (u_2), analyst-wise uncertainty associated with precision (u_3), day-wise uncertainty associated with accuracy/bias (u_4), and analyst-wise uncertainty associated with accuracy/bias (u_5). The combined uncertainty (U) was calculated as follows:

$$U = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2}$$

The combined uncertainty (U) was reported in relative measures as expanded uncertainty, which is twice the value of the combined uncertainty. Relative uncertainty represents the ratio of uncertainty value at a given concentration to the concentration at which the uncertainty is calculated.

3. Results and discussions

3.1. Sample preparation method optimization

Considering the high fat content of Cobia (Liu et al., 2009), a small sample size of 5 g was used for extraction to minimize the co-extractives (fats/lipids) without compromising the method performance. The extraction of fish matrix with cyclohexane:ethyl acetate (9:1; v/v) gave low recovery (<40%) for the several test analytes like fenobucarb, 3-hydroxy carbofuran, trifluralin, α-HCH, β-HCH, lindane, heptachlor, pentachloroaniline, aldrin, fenitrothion, tetraconazole, 4,4-DDMU, fipronil, fipronil sulfone, α-endosulfan, β-endosulfan, and trans-chlordane. Ethyl acetate and acetonitrile extracts gave similar recoveries and were above 60% for all the test compounds, but the matrix effect for ethyl acetate was relatively greater. Hence, acetonitrile was chosen as the extraction solvent. A traditional QuEChERS based sample preparation with dSPE cleanup (50 mg PSA + 50 mg C18) mentioned earlier for fish matrix (Rawn et al., 2010) was also tried, but it could not effectively remove the co-extractives and as a result significant matrix effects (as high as 300%) were recorded for several compounds.

3.2. Cleanup optimization

To examine the nature of the co-extractives, the acetonitrile extract of the fish meat (without dSPE cleanup) was screened by GC-QTOF-MS under the same chromatographic condition in full scan mode. The matrix components were identified based on their accurate mass. We observed that the early and mid-eluting compounds from matrix are mainly fatty acids and triglycerides, whereas the late eluting compounds were mainly cholesterol and related compounds (Table 1).

Based on the above information, a strategy involving three phase separation among water, acetonitrile and hexane was adopted to remove the co-extracted fats and lipids by partitioning into a hexane layer. The volume of hexane used (2 mL) was carefully validated. A higher amount of hexane (e.g. 5 mL) did not reduce the lipid co-extractives in the acetonitrile layer any further, but instead reduced the recovery of OCs and PAHs (e.g. average 30–60% recovery for naphthalene, α-HCH, β-HCH, lindane, heptachlor, anthracene, δ-HCH, aldrin etc.). A comparison of percentage recovery of selected test analytes with 5 and 2 mL of hexane is presented in Appendix B. Further cleanup optimization was designed in such a manner so as to compare with similar existing cleanup techniques (Munaretto et al., 2013; Norli et al., 2011; Rawn et al., 2010).

Table 1
Matrix components co-eluting with the analytes with high matrix effect.

Analytes	RT (min)	ME (%)	Matrix compound detected at corresponding region by GC QTOF	Molecular weight of identified compound
Matrix induced signal suppression				
Naphthalene	5.06	−34	Estragole	148.0883
Phorate	12.03	−72	Octadecane	254.2968
Pentachloroaniline	14.74	−69	Methyldibenzothiophene	198.0498
Chlorothalonil	16.64	−32	Hexadecanoic acid, methyl ester	227.2006
Parathion	19.18	−89	Hexadecanoic acid, ethyl ester	284.271
Tetraconazole	19.90	−99	9,12-Octadecadienoic acid	280.2397
Chlorfenvinphos I	21.01	−31	9,12-Octadecadienoic acid	280.2397
S-Bioallethrin	24.1	−52	Octadecanoic acid, 3-hydroxy-, methyl ester	313.2737
Edifenphos	26.45	−48	3,4′,5,6′-tetra-tert-butylbiphenyl-2,3′-diol	410.3179
Cypermethrin IV	32.10	−39	Squalene	410.3907
Fenvalerate	33.58	−39	Cholesterol	386.3543
Matrix induced signal enhancement				
δ-HCH	15.20	63	Methyldibenzothiophene	198.0498
Penconazole	21.30	59	9,12-Octadecadienoic acid	280.2397
Fipronil	21.35	58	9,12-Octadecadienoic acid	280.2397
Bifenthrin	28.13	83	Squalene	410.3907
Deltamethrin	28.41	71	Cholesteroyl-oleate	368.3438
Fenpropathrin	28.41	71	Cholesteroyl-oleate	368.3438
Cypermethrin II	32.00	54	Cholesteroyl-oleate	368.3438

Freezing the extract with an objective to remove lipid co-extractives at a low temperature significantly reduced the recovery of OCs and PAHs. Hence, in the current procedure, although an aliquot of the middle layer of acidified acetonitrile (1.5 mL) was pipetted out and kept at −20 °C for 20 min to avoid loss of thermo-labile analytes (temperature increased due to dissolution of $CaCl_2$), the lipid precipitates after freezing were not removed. dSPE cleanup of the acetonitrile extracts (1.5 mL) was carried out in triplicate using two different approaches namely 50 mg PSA + 100 mg C18 and a two-step sequential cleanup with100 mg $CaCl_2$ and then with 50 mg PSA + 100 mg C18.

The cleanup achieved using $CaCl_2$ followed by dSPE with 50 mg PSA + 100 mg C18 showed improved recoveries for some compounds such as atrazine des ethyl, atrazine des isopropyl, atrazine, etc. than the cleanup with only PSA-C18. However, it was still not enough to significantly minimize the matrix interferences. Matrix related suppression and enhancements in signals were mainly observed for the test analytes eluting between 5–12 min, 14–24 min, and 25–37 min. To remove the fatty acid co-extractives, a dSPE cleanup step for the acetonitrile (1.5 mL) extract with $CaCl_2$ was introduced in sequence with another succeeding clean up with 50 mg PSA + 50 mg florisil + 150 mg C18. This combination of dSPE sorbents could significantly remove matrix related interferences. A comparison of percentage recovery of selected test analytes with three cleanup approaches is presented in Fig. 1. The recoveries for several OCs (43–118%) and PCBs (26–65%) were quite low as compared to the method described in this paper (60–120%). Munaretto et al. (2013) used 25 mg PSA and 125 mg C18 for dispersive cleanup of 1 mL acetonitrile extract. Clearly this could not remove matrix effect significantly as shown in Fig.1. Norli et al. (2011) used PSA and $CaCl_2$ for dispersive cleanup, but our results indicate that the optimized cleanup method reported in this paper gives a better reduction in matrix effect as compared to PSA + $CaCl_2$ treatment only (Fig.1,). The cleaned acetonitrile supernatant was evaporated, reconstituted in ethyl acetate and injected into GC–MS in full scan mode and overlaid with the total ion chromatogram (TIC) of the acetonitrile extract (reconstituted in ethyl acetate) without cleanup. The overlaid TIC (Fig.2) showed significant removal of co-extractives between 19 and 37 min. Selective removal of co-extractives in dSPE without compromising the recovery of the target analytes requires careful optimization of solid phase sorbents (Banerjee et al., 2009). Florisil (magnesium silicate) and PSA, because of their basic surface remove fatty acid whereas C18 removes nonpolar lipid co-extractives. Often it has been observed that these sorbents remove co-extractives more effectively when used in combination than individually (Gonzalo-Lumbreras, Sanz-Landaluze, & Cámara, 2014; Wilkowska & Biziuk, 2011). Here removal of mid and late eluting fatty acids, triglycerides and sterols could be attributed to the cleanup effect of florisil and addition of increased amount of C18 (150 mg). Hence, the dSPE cleanup was carried out with 100 mg $CaCl_2$ followed by 50 mg PSA + 50 mg florisil + 150 mg C18 in presence of 150 mg $MgSO_4$. Examples of extracted ion chromatogram (XIC) of MRM ions for few mid and late eluting

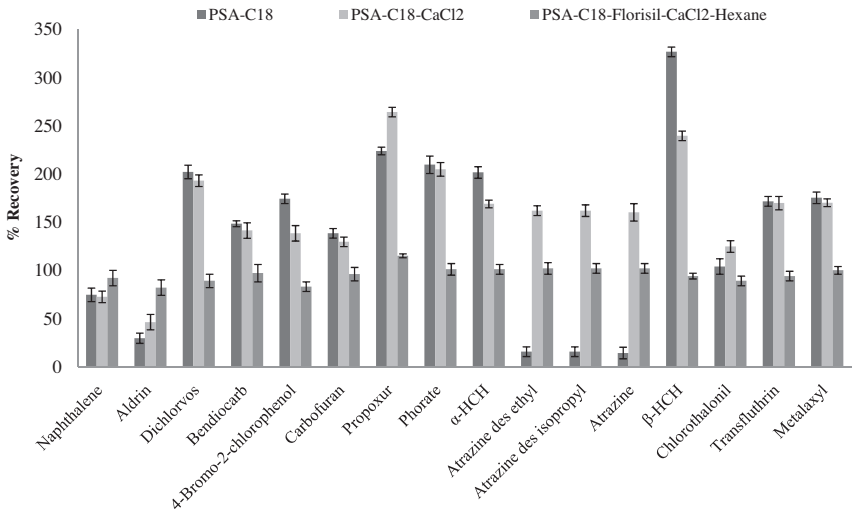

Fig. 1. Percentage recovery (calculated against solvent standard) of selected test analytes for different cleanup approaches.

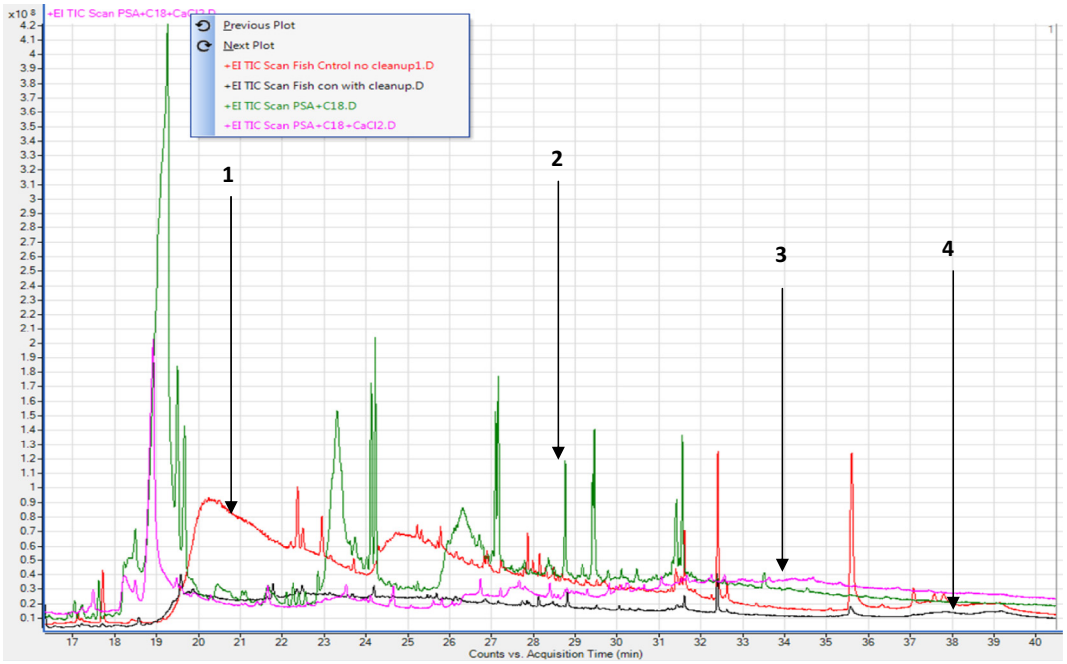

Fig. 2. Comparison of different cleanup approaches showing effect of cleanup on removal of matrix co-extractives where 1: Total Ion Chromatogram (TIC) of control fish matrix without any cleanup; 2: TIC of control fish matrix with PSA-C18 cleanup; 3: TIC of control fish matrix with PSA-C18-$CaCl_2$ cleanup; 4: TIC of control fish matrix with the final optimized cleanup (PSA-C18-Florisil-$CaCl_2$-Hexane).

compounds in unspiked matrix blank and the matrix spiked with standard have been shown in Fig. 3 to demonstrate the good cleanup effect.

The optimized method was also compared with other SPE cleanup techniques commonly used for removal of lipid co-extractives, namely, silica mini column, H_2SO_4 treated silica cartridge and C18 cartridge in sequence to QuEChERS extraction (LeDoux, 2011). On comparison of the GC–MS chromatograms in full scan mode, it is concluded that only the H_2SO_4 treated (50%) silica cartridge cleanup could show better removal of lipid co-extractives as compared to the developed method (Appendix C). However, in this case recovery of OCs were poor (<40%) (Fig. 4) due to degradation in acid treated silica cartridge. Silica mini column cleanup and C18 cartridge cleanup also resulted in lower recovery as compared to the optimized method.

3.3. Method validation

3.3.1. Calibration, accuracy and precision

Good linearity ($r^2 \geqslant 0.99$) was obtained for all the test compounds for both solvent and matrix matched standards (Appendix A). The LOD and LOQ of the test analytes were within 0.001–0.004 and 0.002–0.013 mg/kg, respectively which are well below the MRL of the respective analytes (http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/MRLs-p, ec.europa.eu/sanco_pesticides/public/index.cfm). The recoveries of the test compounds at 10, 25 and 50 μg/kg were within 60–127% with an associated RSD $\leqslant$ 11% (Appendix D). A lower recovery observed for some PAHs (indeno [1,2,3-cd]pyrene, benzo[g,h,i]perylene, dibenz[a,h]anthracene, benzo[b]fluoranthene, Chrysene), a few OCs (2,4-DDE, 4,4-DDE) and pyrethroids (permethrin II) could be attributed to their partial

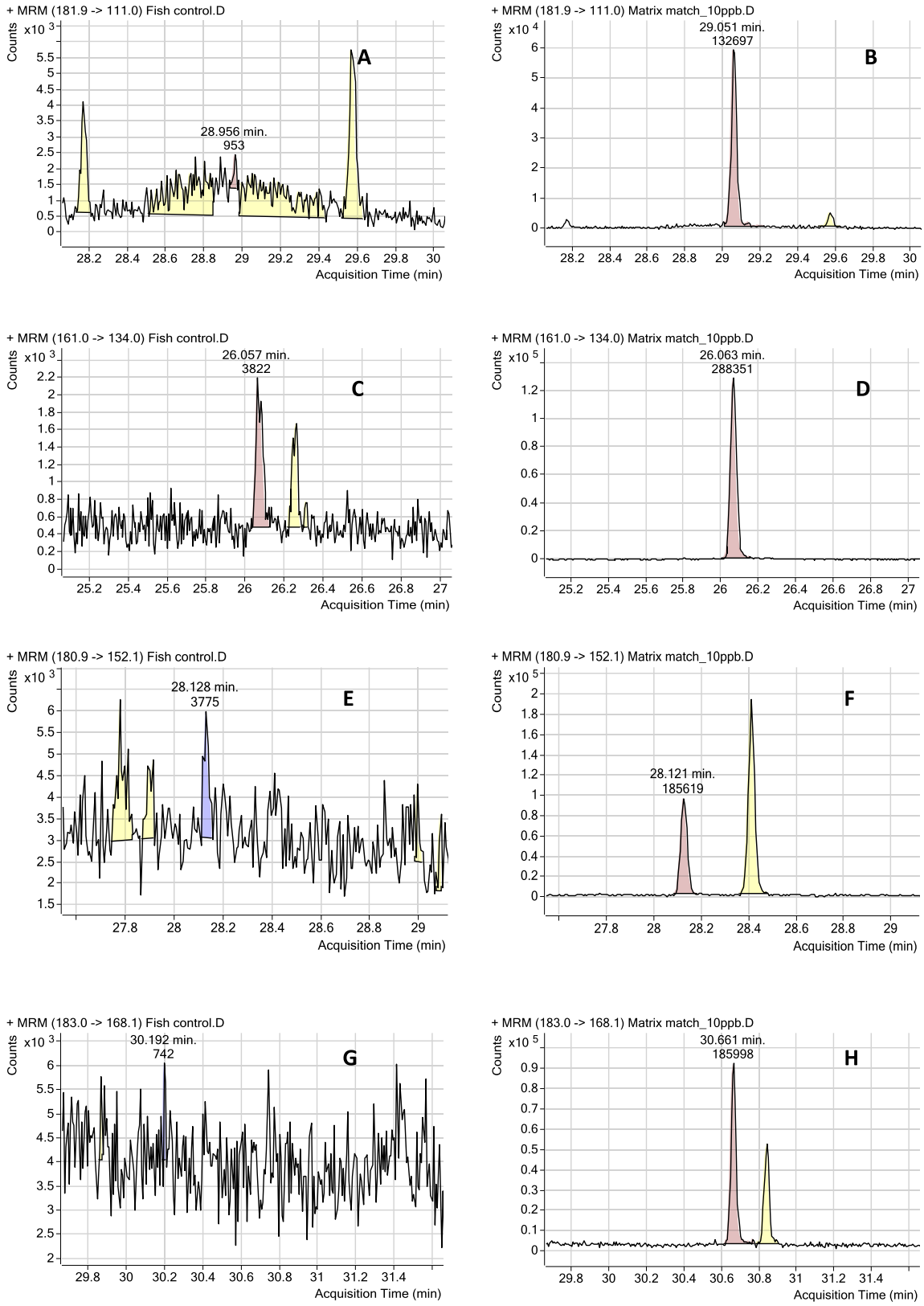

Fig. 3. Extracted ion chromatogram (XIC) of MRM ions of unspiked matrix blank and the matrix spiked with standard for Phosalone (A and B), Triazophos (C and D), L-Cyhalothrin (E and F) and Permethrin I (G and H) respectively.

loss in the hexane layer. Hence, the low recoveries in certain cases were accepted as a compromise since the estimations were satisfactorily repeatable (precision RSD < 10%, $n = 6$). The precision values of the test compounds calculated at 25 μg/kg by the Thompson equation were within 0.08–1.26 (reproducibility RSD) and 0.08–1.24 (repeatability RSD) for most of the compounds, except

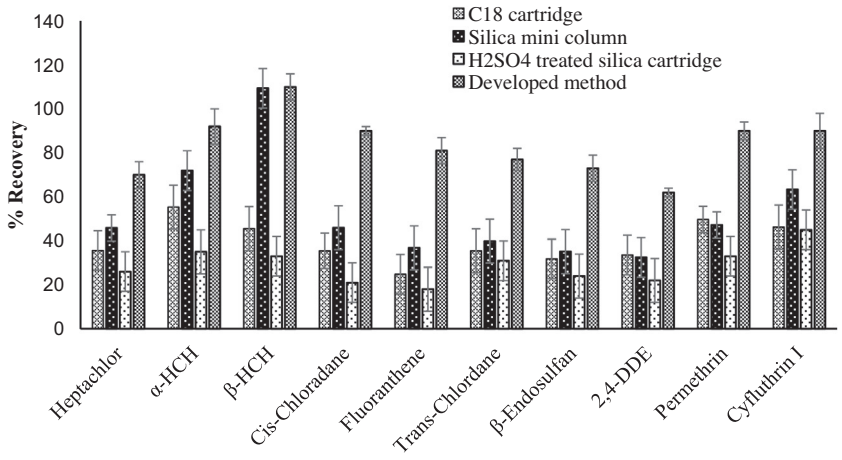

Fig. 4. Recoveries (%) of selected analytes with the developed method as compared to C18 cartridge cleanup, silica mini column cleanup and H_2SO_4 treated silica cartridge cleanup approaches.

for monocrotophos, paraoxon methyl, carbaryl, phosphamidon, malaoxon, captan and captafol, where the reproducibility and repeatability RSDs were more than 2. This is due to their relatively poor chromatographic performance and inherent instability. Some of these compounds are oragnophosphorous and carbamates which are not ideally suited for GC analysis. Captan and captafol are prone to degradation during GC injection which could be the possible reasons for their relatively poor analytical performance (Savant et al., 2010). To ensure the robustness of the method, the recovery experiment at 0.05 mg/kg was performed ($n = 6$) in pangasius catfish (*Pangasianodon hypophthalmus*) having ~3.5% fat. The recoveries obtained for test analytes were within 60–115% with RSDs less than 15%.

3.3.2. Matrix effect

The matrix effect results were grouped into the following 5 categories: high signal suppression (ME < −50%), moderate suppression (ME < −10 to −50%), no matrix effect (ME > −10 to <10%), moderate signal enhancement (ME > 10 to <50%) and high signal enhancement (ME > 50%). Higher matrix effects in terms of signal suppression were noted for 8 (6.7%) out of 119 test analytes whereas for 41 (34.5%) analytes, the signal suppressions were moderate. Similarly moderate signal enhancement was observed for 36 (30.3%) analytes and 20 (16.8%) analytes showed high signal enhancement. No matrix effect was observed for 14 (11.8%) analytes. Similar signal suppressions up to 86.9% were reported by Yu and Xu (2012) in a tea matrix. The method performed better when matrix effects of some of the analytes were compared with that in similar competing methods. Matrix effect of β-HCH, δ-HCH, chlorpyrifos, chlorpyrifos methyl, malathion, β-endosulfan, endosulfan sulfate and DDT was calculated as 32, 63, 13, 11, 13, 19 and 11% as compared to 175.7, 219.4, 85.8, 143.3, 281.5, 83.8 and 156.7%, respectively, reported in literature (Munaretto et al., 2013). Though the method of Sapozhnikova and Lehotay (2013) dealt with a different set of analytes, the range of matrix effect was in general comparable. In this case matrix related signal suppression was observed for 41.2% of the analytes. In order to identify the source of matrix effects, the solvent and matrix matched standards at 10 and 100 μg/L were analyzed using different SRM transitions, and the effect of the selectivity of SRM on minimization of the matrix effect was evaluated. It was observed that choosing a different SRM does not have any significant effect on minimization of matrix effect (examples presented in Table 2). From this data, it was concluded that the probable source of matrix effect could be the active sites in GC. Thus, for quantification of the residues, matrix matched calibration standards were employed. Chromatographic stability of the PTV injector and column was checked for 300 injections over which a stable response was recorded for most of the analytes without changing the injector liner.

Table 2
Effect of selection of different SRM on minimization of the matrix effect (ME%).

Bifenthrin		Heptachlor	
SRM	ME (%)	SRM	ME (%)
181.2 > 165.2	84	271.7 > 236.9	78
181.2 > 166.2	83	236.9 > 118.8	76
165.2 > 115.1	82	236.9 > 142.9	76
166.2 > 115.1	81	273.7 > 236.9	78
166.2 > 165.2	81	273.7 > 238.9	78
182.2 > 166.2	82	336.6 > 265.7	81
α-HCH		Phorate	
SRM	ME (%)	SRM	ME (%)
180.9 > 145.0	56	121.0 > 65.0	74
180.9 > 109.0	56	121.0 > 47.0	74
182.9 > 109.0	57	128.9 > 47.0	76
182.9 > 147.0	55	128.9 > 65.0	76
216.9 > 145.0	60	230.9 > 174.8	75
216.9 > 181.0	58	230.9 > 128.9	75

3.3.3. Measurement uncertainty

Uncertainty of measurement for the test analytes estimated at 25 μg/kg was within 12–22%, indicating ruggedness of the developed method. The uncertainty associated with u_1 was less than 4%, and those for u_2 and u_3 were less than 5%. In case of u_4 and u_5, the uncertainties were slightly higher (up to 7%). The uncertainty was higher than 30% for those compounds having reproducibility and repeatability RSDs more than 2 (monocrotophos, paraoxon methyl, carbaryl, phosphamidon, malaoxon, captan and captafol). Thus, it was concluded that most of these compounds are more suitable for liquid chromatographic analysis as reported in the literature (Banerjee et al., 2007). For the analysis of captan and captafol, different sample preparation approaches might help as these compounds are known to be difficult to analyze (Yu & Xu, 2012).

4. Conclusions

The developed multiresidue method was successful for the analysis of 119 contaminants in high fatty matrix with satisfactory precision and accuracy. When compared to the reported methods in literature, the developed multiresidue method showed clear sample cleanup efficiency and wider scope as demonstrated by satisfactory analysis (recovery, accuracy, precision) of multiclass pesticides and other contaminants. Simultaneous analysis of multiclass contaminants in a complex matrix is a challenging task considering their eclectic interaction and co-elution with matrix components (Jadhav, Oulkar, Ahammed Shabeer, & Banerjee, 2015). The satisfactory recoveries in different fish matrix demonstrated suitability of the method for analysis of pesticide and PAH residues in other varieties of fish. It was possible for a single person to prepare approximately 20 samples in three hours. Extraction of the analytes with acetonitrile in presence of hexane was helpful in minimizing the co-extracted matrix components. The cleanup of the acetonitrile extract (1.5 mL) with $CaCl_2$ followed by cleanup with 50 mg PSA + 50 mg florisil + 150 mg C18 was effective in significantly minimizing the matrix effects. The method could be successfully applied for routine laboratory analysis of pesticide and PAH residues in fish samples without compromising long term stability of the analytical instrument.

Acknowledgement

The authors acknowledge funding support from the Indian Council of Agricultural Research (ICAR) National Fellow project and the National Referral Laboratory project of Agricultural and Processed Food Products Export Development Authority (APEDA). Thanks are also due to Agilent Technologies, Bangalore for helping us in carrying out the GC-QTOF MS analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.09.014.

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